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Title:	Method for Culturing Avian Spermatogonial Stem Cells and Avian Spermatogonial Stem Cells Prepared Thereby		

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DECLARATION UNDER 37 C.F.R. § 1.132

I declare:

1. I, Jae Yong Han the chief of the inventors of United State Patent application serial No. 10/569,847 declare as follows:

2. The present invention of the method for long term culture of spermatogonial stem cells (SSCs) from avian testes satisfies the long felt but unresolved need. The long felt but unresolved need in the field the present invention pertains to is to prepare transgenic chicken with high efficiency.

3. SSCs have an ability to self-renew and to produce large numbers of differentiating germ cells that become spermatozoa throughout postnatal life and transmit genetic information to the next generation. In vitro manipulation of SSCs offers the opportunity to study the mechanisms of continuation of the germline and to develop novel techniques for germline modification or therapy and producing of transgenic animal. In other words, SSCs can be utilized in the generation of transgenic animal or gene therapy of sperm cells through the introduction of gene into SSCs and

transplantation of genetically manipulated SSCs into the seminiferous tubules of recipient. It also can be useful tools to study the spermatogenesis.

4. Recent research with SSCs in the mouse demonstrates the potential of these stem cells as a centerpiece in a new era of clinical application for treatment of infertility and regenerative medicine (Brinster RL and Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* **91:** 11298–11302; Brinster RL and Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci USA* **91:** 11303–11307; Kubota H *et al.* (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* **101:** 16489–16494).

5. Furthermore, a system to transplant mouse testis cells from a fertile donor male to the seminiferous tubules of an infertile recipient male has been developed. Spermatogenesis is generated from transplanted cells, and recipients are capable of transmitting the donor haplotype to progeny. After transplantation, primitive donor spermatogonia migrate to the basement membrane of recipient seminiferous tubules and begin proliferating. Eventually, these cells establish stable colonies with a characteristic appearance, which expands and produces differentiating germ cells, including mature spermatozoa. Thus, the transplanted cells self-renew and produce progeny that differentiate into fully functional spermatozoa.

6. As a bioreactor, bird such as chicken has been proved as the most efficient system for producing useful therapeutic proteins. More than half of the egg white protein contents derive from the ovalbumin gene and other four proteins (lysozyme, ovomucoid, ovomucin and conalbumin) present at levels of 50 milligrams or greater. In addition, the naturally sterile egg contains egg white protein at high concentration allowing for a long shelf life of recombinant protein with out loss in activity. Transgenic chicken as a bioreactor for production of recombinant therapeutic

proteins has advantages such as low cost, rapid development of production flock and beneficial glycosylation profile of the protein obtained from the eggs.

7. In spite of the above-mentioned advantages, transgenic procedures in the bird have lagged far behind other vertebrates because of its unique process of early gamete and embryo development compared with other animals such as mouse.

8. The technique of long term culture of chicken SSCs makes it possible to efficiently create transgenic chickens that produce useful recombinant proteins through the procedure of transfecting the cultured SSCs with a recombinant gene construct and subsequent transplanting the transfected SSCs into the testes of recipient chicken. This system consists of isolation and in vitro-culture of chicken testicular cells, i.e. SSCs, transfer of in vitro-maintained SSCs into heterologous testes, production of germline chimeras and cross-breeding of them for confirming the presence of heterologous, functional spermatozoa differentiated from donor SSCs.

9. There are several significant advantages of SSCs over PGCs for preparing transgenic chickens.

10. First, germline stem cells of SSCs can be obtained in large quantities compared to the conventional method whereby embryo-derived primordial germ cells (PGCs) are used.

11. Second, the preparing method of transgenic chicken using SSCs takes less time to obtain transgenic progeny chicken originated from transplanted SSCs compared to the method utilizing PGCs. In the method using PGCs, PGCs are introduced into a blood vessel of embryo and the embryo injected with PGCs has to be incubated, hatched and brought up to the adult chicken, and then, progeny transgenic chicken is produced through cross-breeding. Thus, compared with the method of using PGCs, the method for preparing transgenic chicken by introducing SSCs into the testes of recipient has the advantages of not requiring the time needed for incubating, hatching

the transgenic embryo and bringing it up to the adult chicken for obtaining transgenic progeny.

12. Third, the preparing method of transgenic chicken using SSCs does not require the procedure of sex selection because it introduces male SSCs into the male recipient. Thus the method using SSCs can eliminate the time and cost required for sex selection. On the contrary, the procedure of sex selection is required in the method using PGCs in order to increase the efficiency of producing transgenic progeny by matching the sex of donor PGCs and recipient.

13. Fourth, the method for producing transgenic chicken by using SSCs can improve the efficiency of transmission of the introduced gene to the progeny because the process of development from SSCs into the intact sperm cells is relatively simple and does not require long time. On the other hand, the chance of transmission of a foreign gene introduced into PGCs to the progeny is relatively low in the method using PGCs because PGCs introduced into an embryo necessarily undergo an extraordinary complex procedure during gonad development.

14. Fifth, the method using PGCs requires the process of bringing up the hatched chick to the adult chicken and carrying out cross-breeding. Thus, compared to the method using PGCs, the method using SSCs can remove the cost and time for incubating and hatching the embryo and rearing the hatched chick to adult chicken because the adult chicken injected with SSCs can be directly used for cross-breeding to prepare transgenic progeny.

15. Thus, the establishment of long term culture of chicken SSCs is essential and fundamental technique for utilization of SSCs to prepare transgenic chicken. The long term culture of mouse SSCs is already established, however, the culture of avian such as chicken SSCs is firstly achieved by the inventors of the instant invention and this method is strongly expected to provide a technological basis to produce transgenic chickens with high economical efficiency.

16. The present invention of the method for long term culture of spermatogonial stem cells

(SSCs) from avian testes cannot be expected from thetechniques that disclose the isolation and culture of SSCs from *mouse* testes.

17. First, birds have internalized testicles as contrary to a mouse having externalized testicles. More specifically, in the mouse, the testes are located under the peritoneal cavity and exposed to exterior of the body. Thus, mouse testes are easily detected with the naked eye. This makes it easy to excise and isolate testicular cells including SSCs from the mouse body. On the contrary to this, the testes of birds are located within the center of the body cavity, ventral to the spine and kidney (See Fig. 8.1. of page 209 of Etches RJ. Reproduction in poultry. Cab International 1996: 208-214), and thus, they cannot be easily observed without surgical process. In addition, *rete testis* is connected to a whole side of a testis, rather than connected to a part of the testes. These render it more difficult to prepare and isolate testicular cells containing SSCs from the bird body compared to the mouse. Accordingly, in order to isolate SSCs from the internalized bird testes, a complex surgical process is essential.

18. Second, the physiological conditions of spermatogenesis in birds are completely different from that of mouse. For example, the spermatogenesis in birds is carried out under the temperature of 40-41 °C which is distinctly different to the spermatogenesis temperature of 24-26°C of the mammal including mouse. The difference of the spermatogenesis temperature in birds and mouse comes from the difference of the location of the testes in the body as mentioned above.

19. Third, if the method for preparing mouse SSCs is directly applied to bird system, the yield of obtaining bird SSCs is very low. The reason for this can be summarized as followings. Many specific markers for mouse SSCs have been already found. However, in bird system, specific markers for SSCs have not been identified and it is difficult to use mouse SSCs markers in

the avian system. Furthermore, it is expected that various characteristics exist in spermatogenesis according to the different age steps of the avian. The specific methods for preparing and isolating avian SSCs must be altered in accordance with the specific age stages of the bird, and the specific aspects of the age stages of the bird are distinctly different from that of mouse. Thus, the method for preparing mouse SSCs cannot be directly applied to the avian system.

20. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Signature: Jaeyong Date: Feb. 3, 2009

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Curriculum Vitae

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2007 - present: Deputy Director, Research Institute for Agriculture and Life Sciences, Seoul National University

2001 - 2003: Head, Department of Animal Science and Technology, Seoul National University

2001 - 2003: Director, University Animal Farm, Seoul National University

2001 - present: Professor, Department of Agricultural Biotechnology, Seoul National University

1996 - 2001: Associate Professor, Department of Agricultural Biotechnology, Seoul National University

1991 - 1996: Assistant Professor, Department of Animal Science and Technology, College of Agriculture and Life Sciences, Seoul National University

1988 - 1991: Research Assistant, Department of Animal Science, University of Minnesota, USA

1984 - 1986: Research Assistant, Seoul National University

o **ACTIVITIES IN PROFESSIONAL SOCIETIES**

2009 - present: Editor-in-Chief, Korean Society of Animal Sciences and Technology

2008 - present: Special Advisory Board, The Journal of Poultry Science (Japan)

2008 - present: Editorial Advisory Board, The Open Reproduction Science Journal

2008 - present: Editor-in-Chief, Animal Biotechnology & Experimental Medicine

2007 - present: Registered Reviewer, Reproduction Biology and Endocrinology

2007 - 2008: Chair of Scientific Committee, Korean Society of Animal Sciences and Technology

2005 - present: Vice President, Korean Society of Poultry Science

2002 - present: Member, Central Agricultural Advisory Communal Consultant, Rural Development Administration

2002 - 2005: Chair, Center for Poultry Research, BioGreen 21 Program, Rural Development Administration

1999 - present: Korea Branch Secretary, World Poultry Science Association

1999 - 2002: Secretary General, Editor, Korean Society of Poultry Science

1999 - 2004: Board of Directors, Korean Society of Animal Sciences and Technology

1991 - present: Editorial Board, Assistant Editor, Asian-Australasian Journal of Animal Science

1996 - 1999: Secretary General, Editor, Korean Society of Animal Genetics and Breeding

1994 - 2000: Editor, Korean Journal of Animal Reproduction

1994 - 2004: Section Editor (Genetic Section), Korean Journal of Animal Science and Technology

○ **LIST OF PUBLICATION (SCI)**

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2. Lee JH, Lee EJ, Lee CH, Lee DJ, Park JH, **Han JY**, Lim JM (2009) Requirement of leukemia inhibitory factor establishing and maintaining of embryonic stem cells in mice. *Fertility and Sterility* (in press).
3. **Han JY** (2009) Germ cells and transgenesis in chickens (invited paper). *Comparative Immunology, Microbiology and Infectious Disease* 32:61-80.
4. Kim KM, Sung S, Caetano-Anolles G, **Han JY**, Kim H (2008) An approach of orthology detection from homologous sequences under minimum evolution. *Nucleic Acids Research* 36:e110.
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9. Rengaraj D, Kim DK, Zheng YH, Lee SI, Kim H, **Han JY** (2008) Testis-specific novel transcripts in chicken: *in situ* localization and expression pattern profiling during sexual development. *Biology of Reproduction* 79:413-420.
10. Shin SS, Kim TM, Kim SY, Kim TW, Seo HW, Lee SK, Kwon SC, Lee GS, Kim H, Lim JM, **Han JY** (2008) Generation of transgenic quail through germ cell-mediated germline transmission. *FASEB Journal* 22:2435-2444.
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12. Park TS, Kim MA, Lim JM, **Han JY** (2008) Production of quail (*Coturnix japonica*) germline chimeras derived

from *in vitro*-cultured gonadal primordial germ cells. *Molecular Reproduction and Development* 75:274-281.

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primordial germ cells *in vivo* and *in vitro* by soft X-ray irradiation. *Animal Reproduction Science* 95:67-74.

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bone marrow cells. *Biotechnology Letters* 25:205-211.

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62. **Han JY**, Park TS, Hong YH, Jeong DK, Kim JN, Kim KD, Lim JM (2002) Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained *in vitro* for an extended period. *Theriogenology* 58:1531-1539.
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